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# **Brain Damage from Soman-Induced Seizures Is Greatly Exacerbated by Dimethyl sulfoxide (DMSO): Modest Neuroprotection by 2-Aminoethyl diphenylborinate (2-APB), a Transient Receptor Potential Channel Inhibitor and Inositol 1,4,5-triphosphate Receptor Antagonist**

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*In conducting the research described in this report, the investigators complied with the regulations and standards of the Animal Welfare Act and adhered to the principles of the Guide for the Care and Use of Laboratory Animals (NRC 1996).*

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## **ABSTRACT**

Soman is a nerve-agent that produces seizures and seizure-related brain damage (SRBD). It is well known that termination of soman-induced seizures, using anticonvulsant drug therapy, would be the ideal means of preventing SRBD. However, these seizures quickly develop into *status epilepticus* and become refractory to anticonvulsant therapy shortly after their onset. Medical care for some battlefield casualties will likely be delayed beyond the therapeutic window of opportunity to terminate soman-induced seizures. Moreover, SRBD that has already been triggered will continue, along the pathological cascade, unabated by the currently fielded therapeutic regimen. Thus, there is a need for adjunct drug therapy that is capable of interrupting ensuing pathology and augmenting neuroprotection when administered, in conjunction

with anticonvulsants, during the refractory phase of soman-induced seizures. Considerable evidence supports a pivotal role of sustained elevations in intracellular calcium (i.e., delayed calcium overload) in SRBD resulting from soman-induced seizures and *status epilepticus*. In addition, there are several reports that a neuroprotective approach, aimed at attenuating delayed calcium overload, combined with standard anticonvulsant therapy, provides greater protection against soman-induced SRBD than anticonvulsant therapy alone. Transient receptor potential (TRP) channels, a new class of membrane ion channels, are responsible for capacitative calcium entry and have been linked to delayed calcium overload. The TRP channel antagonist, 2-aminoethyl diphenylborinate (2-APB), blocks calcium capacitative entry and is reported to be neuroprotective in various excitotoxic models. 2-APB is also a potent inositol 1,4,5, triphosphate (IP3) receptor antagonist and blocks release of calcium from internal stores. In the present study, we assessed the neuroprotective efficacy of 2-APB against soman-induced SRBD. Our results indicate that a single injection of 2-APB (5.0 or 13.7 mg/kg; i.p.), 5 min following seizure onset, had modest neuroprotective efficacy. However, these findings were obscured by unexpected neuropathological effects of the vehicle, dimethyl sulfoxide (DMSO), which greatly exacerbated soman pathology. Of considerable relevance, the present study is the first to attempt *in vivo* neuroprotection, using 2-APB, in any species. This is also the first attempt at neuroprotective, against soman-induced SRBD, either by blocking TRP channels or blocking the intracellular mediator of muscarinic receptor over stimulation by soman (i.e., IP3 second messenger receptors).

## INTRODUCTION

Protection against brain damage resulting from nerve agent exposure is of significant military concern. The current regimen of antidotal therapy most effectively addresses the acute life-threatening consequences of exposure. However, many soldiers surviving the initial life-threatening effects of nerve agent intoxication are likely to develop seizures. This is particularly true, of unprotected civilians, in the event of a terrorist attack. Anticonvulsants such as diazepam can arrest soman-induced seizures when administered within 40 min following onset; however, after that time, seizures become refractory to anticonvulsant therapy (e.g., Lipp, 1972, 1973; Shih, 1990; Shih *et al.*, 1991; Capacio and Shih, 1991; Philippens *et al.*, 1992; Sparenborg *et al.*, 1993; McDonough and Shih, 1993; Harris *et al.*, 1994; Shih *et al.*, 1999; Lallement *et al.*, 2000; McDonough *et al.*, 2000). Unless seizures are terminated, the currently fielded therapy does not afford complete protection against brain damage. Furthermore, it is very likely that there will be unconscious nerve agent exposure victims who suffer silent seizures. Since these seizures are not associated with the usual behavioral manifestations seen with typical seizures, victims may not receive any anticonvulsant treatment. If left untreated, or if they cannot be arrested, nerve agent-induced seizures progress to *status epilepticus* and lead to extensive brain damage. Therefore, there is a clear need for adjunct therapy that is capable of preventing or reducing brain damage even if seizures have progressed to *status epilepticus* and can not be terminated. An effective neuroprotectant would

greatly increase the window of opportunity for therapeutic intervention and augment the beneficial effects of anticonvulsants in the currently fielded regimen.

Soman (O-1,2,2-trimethylpropylmethyl-phosphonofluoridate) is an organophosphorus nerve agent that produces *status epilepticus* and seizure-related brain damage (SRBD) (Petras, 1981; Lemercier *et al.*, 1983; McLeod *et al.*, 1984; McDonough *et al.*, 1987). Seizure induction results from soman's ability to irreversibly inhibit acetylcholinesterase (AChE), causing an elevation in acetylcholine concentration in the brain (reviewed in Solberg and Belkin, 1997). Once initiated by elevated acetylcholine concentrations in susceptible brain regions, seizures are maintained by excess glutaminergic synaptic transmission (Olney *et al.*, 1983; Sparenborg *et al.*, 1992; Solberg and Belkin, 1997). It is well known that glutamate receptor overstimulation produces excitotoxicity and neuronal cell death. Moreover, the mechanism by which glutamate excitotoxicity causes neuronal death is dependent on sustained elevations in intracellular free calcium (i.e., delayed calcium overload) (Olney *et al.*, 1983, 1987; Choi, 1987, 1988; Randal and Thayer, 1992). There is considerable evidence that soman-induced seizure-related brain damage (SRBD) results from glutamate excitotoxicity and the ensuing delayed calcium overload. For example, it has been reported that SRBD from soman can be alleviated by administration of various N-methyl-D-aspartate (NMDA) receptor antagonists: MK-801, GK-11, TCP, HU-211, and Ketamine (Olney *et al.*, 1983; Braitman and Sparenborg, 1989; Shih, 1990; Sparenborg *et al.*, 1992; McDonough and Shih, 1993; Lallement *et al.*, 1993, 1994, 1997; Solberg and Belkin, 1997; Filbert *et al.*, 1999; Carpentier *et al.*, 2001a, 2001b, De Groot *et al.*, 2001; Dorandeu *et al.*, 2005; Filbert *et al.*, 2005). The NMDA ionotropic receptor is a ligand-gated calcium channel, and the above antagonists counteract delayed calcium overload by blocking calcium influx. Unfortunately, NMDA receptor antagonists have the drawback of being neurotoxic themselves. Neurotoxicity in the posterior cingulate and retrosplenial cortices has been reported following their use (Olney *et al.*, 1989; Fix, 1994; Wozniak *et al.*, 1998). Additional evidence indicates that a sizable portion of the intracellular free calcium, contributing to neuronal pathogenesis, comes from intracellular stores (i.e., endoplasmic reticulum, ER) (Randal and Thayer, 1992; Mody and MacDonald, 1995; Yoon *et al.*, 1996; Wei and Perry, 1996; Neibauer and Gruenthal, 1999; Pelletier *et al.*, 1999; Yu *et al.*, 1999; Nakayama *et al.*, 2002; Verkhratsky and Toescu, 2003). The ryanodine receptor antagonist, dantrolene, has been shown to diminish soman-induced SRBD by blocking calcium release from the ER (Ballough and Filbert, 2003; Filbert *et al.*, 2005). Additional evidence of neuroprotection by ryanodine receptor antagonists (i.e., cannabinoids) is reported by Zhuang *et al.* (2005). The most widely studied mediator of intracellular calcium mobilization is the second messenger inositol 1,4,5-triphosphate (IP3). This second messenger is pivotal in muscarinic acetylcholine receptor (mAChR) receptor signaling and seizure induction by soman (Savolainen and Hirvonen, 1992; Bodjarian *et al.*, 1993). It is also elevated by the subclasses of metabotropic glutamate receptors (mGluRs) that have been shown to contribute to glutamate excitotoxicity (Ferraguti *et al.*, 1997; Dale *et al.*, 2000).

Recent evidence points to the involvement of a new class of membrane ion channels in the mediation of calcium flux responsible for delayed calcium overload and

neuronal cell death. Transient receptor potential (TRP) channels have been referred to as "the last bastion of ion channels" (Clapham *et al.*, 2001). They are also considered the most likely ion channel candidates responsible for "capacitative calcium entry" (reviewed in Putney, 2003). Capacitative calcium entry is a process whereby the depletion of calcium from intracellular stores (e.g., via IP<sub>3</sub> or ryanodine receptor activation) signals the opening of calcium permeable TRP channels, in the plasma membrane, that remain open until internal calcium stores are completely replenished. Thus, TRP channels provide a means for inducing prolonged and sustained elevations in intracellular calcium (Putney, 2001; Putney, 2003). In addition, TRP channels have been linked to delayed calcium overload resulting from glutamate excitotoxicity and neuronal cell death (Aarts *et al.*, 2003; Chinopoulos *et al.*, 2004; Moran *et al.*, 2004).

There is considerable evidence that 2-aminoethyl diphenylborinate (2-APB) inhibits TRP channel-mediated capacitative calcium entry, as well as IP<sub>3</sub> ionotropic receptors that mediate intracellular calcium release (e.g. Maruyama *et al.*, 1997; Missiaen *et al.*, 2001; Kukkonen *et al.*, 2001; Zhou *et al.*, 2007). As a point of clarification, "2-APB" (or 2APB) correctly refers to 2-aminoethyl diphenylborinate or 2-aminoethoxy diphenylborane (Chawla *et al.*, 2001); however, it has been misleadingly called "2-aminoethoxy diphenylborate" in much of the literature. There is also good evidence that 2-APB can alleviate excitotoxic neuronal damage. In 2004, Chinopoulos *et al.* reported that delayed calcium entry in cortical neurons, resulting from excitotoxic insult, is diminished by 2-APB. Recent findings by Lipski *et al.* (2006) indicate that 2-APB reduced swelling and delayed calcium overload in CA1 hippocampal neurons subjected to oxygen-glucose deprivation (an *in vitro* model of stroke). In addition, Tang *et al.* (2005) reported that 2-APB protects against calcium overload induced neuronal apoptosis, in murine spiny neurons (i.e., an *in vitro* model of Huntington's disease).

The present study was undertaken as a preliminary step in determining the possible neuroprotective efficacy of 2-APB, against brain damage resulting from soman-induced seizures and *status epilepticus*, and its suitability for advancement to the next phase of experimentation. For reasons stated above, a candidate's ability to enhance neuroprotection when co-administered with anticonvulsant therapy (e.g., diazepam), more than 40 minutes following seizure onset, is most relevant. However, when assessing a new test drug, that has a completely novel mechanism of action, it has been our practice (e.g., Filbert *et al.*, 1999; Meier *et al.*, 1999) to first examine the candidate's ability to protect in a less severe model of soman-induced SRBD. By administering the candidate 5 min following seizure onset, we maximize its opportunity to disrupt the pathological cascade, and/or exert an anticonvulsive effect before *status epilepticus* becomes established. While it might appear more efficient to start by testing a new candidate in the circumstance of its anticipated use (i.e., 40 min following seizure onset, in conjunction with diazepam), the greatly increased severity of insult could overwhelm a potential neuroprotective efficacy of the candidate alone. If the latter were to occur—concomitant to increased neuroprotection by combined candidate+diazepam, compared to diazepam alone—it would be impossible to discern the candidate's effect, e.g., potentiating, synergistic, etc. In our experience, these possibilities are not merely theoretical. To avoid these complications, our laboratory employs a two-phase paradigm

that first evaluates a candidate's individual effectiveness when administered 5 min after seizure onset (Filbert et al., 1999; Ballough and Filbert, 2003). In the second phase, promising candidates are tested at 40 min in conjunction with diazepam. Insufficient efficacy at 5 min abrogates the necessity of subsequent testing. Unfortunately, the latter applies to the present results obtained with 2-APB. These results also demonstrate augmented neurotoxicity by the dimethylsulfoxide (DMSO) vehicle.

## METHODS

Eighty male Sprague-Dawley rats (CRL: CD[SD]-BR; Charles River Labs, Wilmington, MA), weighing between 250-300 g, were used. Animals were housed individually in polycarbonate cages under conditions of constant temperature ( $21 \pm 2^{\circ}\text{C}$ ) and humidity ( $50 \pm 10\%$ ), using at least 10 complete air changes per hr of 100% fresh air, and a 12-hr light-dark cycle (full spectrum lighting cycle with no twilight). Throughout the study, food and water were available *ad libitum*, except during the observation period, which began 1.5 hr prior to and ended 6 hr following soman administration.

Representative animals, from each group, were randomly selected for electrocorticographic (ECoG) recordings. These rats were anesthetized with sodium pentobarbital (50 mg/kg, diluted in saline to give an intraperitoneal [i.p.] injection volume of 3.3 mL/kg) and positioned in a stereotaxic apparatus (David Koff Instruments, Tujunga, CA); for most subjects, supplemental injections of dilute pentobarbital (i.e.,  $10 \pm 3$  mg/kg) were given prior to completion of surgeries, as needed. Prior to incisions, subcutaneous (s.c.) xylocaine was administered for local analgesia. Placement of screw electrodes was performed in accordance with the procedure recommended by Braitman and Sparenborg (1989) for ECoG recordings. Electrodes were connected to a standard small-animal head-piece and secured by dental cement.

On the morning of the fifth or sixth day following surgeries, electrode-implanted animals were connected to an ECoG recording system and allowed at least 30 min to acclimate. Baseline ECoG activity and behavior were monitored for at least 15 min. Following baseline recordings, animals were injected (i.p.) with 125 mg/kg of the oxime HI-6. This was followed 30 min later by injection of 180  $\mu\text{g/kg}$  soman (1.6 LD<sub>50</sub>, s.c.) or sterile saline. Within one min following soman or saline injection, animals were injected intramuscularly (i.m.) with 2 mg/kg atropine methylnitrate (AMN). All rats, with the exception of those belonging to the untreated control group, received HI-6 and AMN. These were employed to protect against the peripheral effects of soman and to reduce mortality without affecting seizures. It is worth noting that AMN rather than atropine sulfate is used because the former does not cross the blood-brain-barrier and will not diminish seizure activity or interfere with central effects of the neuroprotective candidate drug. Seizure onset, following soman administration, corresponded to the initiation of sustained ECoG amplitudes greater than four times baseline (e.g., McDonough and Shih, 1993). For the remaining rats, not instrumented for ECoG recordings, seizure onset times were inferred from overt manifestations, e.g., Straub tail (see below). Five minutes following seizure onset, 2-APB (Sigma-Aldrich Co.), or vehicle (ultra pure dimethyl

sulfoxide, DMSO; Sigma-Aldrich Co.), was administered to experimental animals (see below). At the end of the 6-hr observation period, each soman-exposed rat received a supplementary injection of isotonic saline (5 mL, i.p.) to prevent dehydration.

Since the present study was the first study to assess the *in vivo* neuroprotective efficacy of 2-APB, it was necessary to extrapolate an approximate dosage from effective *in vitro* concentrations. By assuming 70% total body water, for a 300 g rat, Chinopoulos (2004) estimated the neuroprotective dosage at 23 mg/kg. Consistent with his recommendation, , we first utilized 22.5 and 10.0 mg/kg 2-APB in DMSO (1.0 mL/kg). After it appeared that either 2-APB or DMSO increased lethality in soman treated rats (see mortality in Table 1), dosages and volumes were adjusted to 13.7 and 5.0 mg/kg 2-APB in 0.7 and 0.5 mL/DMSO, respectively. Treatment groups are listed below.

#### Major Groups:

1)	Soman-positive controls	n = 30
2)	Soman + 2-APB (13.7 mg/kg) in DMSO (0.7 mL/kg)	n = 11
3)	Soman + 2-APB (5.0 mg/kg) in DMSO (0.5 mL/kg)	n = 10
4)	Soman + DMSO (0.7 mL/kg)	n = 6
5)	Soman + DMSO (0.5 mL/kg)	n = 4
6)	Untreated controls	n = 6

#### Pilot Groups:

1)	Soman + 2-APB (22.5 mg/kg) in DMSO (1.0 mL/kg)	n = 3
2)	Soman + 2-APB (10.0 mg/kg) in DMSO (1.0 mL/kg)	n = 3
3)	Soman + DMSO (1.0 mL/kg)	n = 4
4)	Non-Soman + 2-APB (22.5 mg/kg) in DMSO (1.0 mL/kg)	n = 3

Twenty-nine  $\pm$  1 hr after soman administration, rats were given a lethal injection of pentobarbital anesthesia (130 mg/kg, i.p.) and euthanized, upon evidence of labored breathing, via transcardial perfusion with ice cold 10% NBF. Brains were excised and post-fixed in 10% NBF for approximately 24 hr prior to tissue processing. Subsequently, brain specimens were paraffin processed and coronally sectioned at 4  $\mu$ m using a rotary microtome. Two brain sections (stereotaxic coordinates between bregma -2.64 mm and -3.48 mm [Paxinos and Watson, 2005]) from each animal were collected for hematoxylin and eosin (H&E) or microtubule associated protein 2 (MAP-2) histo- or immunohistochemical processing, respectively. H&E stained brain sections were assessed for classical histopathological damage to the piriform cortex. Damage was scored on a scale of 0 to 4, where 0 = no histologic lesion, 1 = minimal damage (1-10% neuronal loss), 2 = mild (11-25% neuronal loss), 3 = moderate (26-45% neuronal loss) and 4 = severe (> 45% neuronal loss). MAP-2 immunostained brain sections were ranked according to severity of temporal lobe necrosis and were further employed for image analysis assessments (see below).

MAP-2 immunohistochemistry utilized a monoclonal antiserum, raised in mice, against microtubule-associated protein 2 (MAP-2) (Sigma Chemical Co., St Louis, MO), and employed the avidin-biotin-peroxidase method of Hsu *et al.* (1981). Morphometric

image analysis of MAP-2 immunostained brain sections was performed using an image analysis system obtained through I-CUBE Inc. (Glen Burnie, MD). This system included Image-Pro Plus v4.1 software, Sony Power HAD camera, Hitachi CM771 monitor and I-CUBE computer. The system was interfaced with an Olympus BH-2 Biological Microscope (Olympus Optical Co., Ltd., Tokyo, Japan). Cross-sectional areas of MAP-2 negative staining (i.e., necrosis [Ballough *et al.*, 1995; Hicks *et al.*, 1995]) in piriform cortex and contiguous brain regions (e.g., amygdaloid nuclei and perirhinal cortex) were measured according to the procedure of Ballough *et al.* (1995). Previous studies have shown that the piriform cortex (including surrounding brain regions) presents the most clearly defined and easily quantifiable lesions of contiguous necrosis following soman-induced seizures in rats (e.g., Ballough *et al.*, 1995).

H&E qualitative damage ratings were grouped according to treatment, and pair-wise comparisons performed using the Mann-Whitney U nonparametric test for comparing the central tendency of two independent samples. Cross-sectional areas of contiguous necrosis (MAP-2 negative areas) were grouped by treatment, and each experimental group mean was compared against the one standard (i.e., the soman control group mean) using the Dunnett's test for multiple comparisons (i.e., Dunnett's ANOVA,  $p < 0.05$ ). For the pair-wise comparison of combined groups, i.e., soman intoxicated animals receiving 2-APB (5.0 - 13.7 mg/kg in DMSO) vs. those receiving the DMSO (0.5 - 0.7 mL/kg) vehicle only, a two sample t-test was used.

## RESULTS

All soman-treated rats exhibited sustained seizures and *status epilepticus* for several hours. Proconvulsive behavioral signs of soman intoxication included repetitive chewing, facial and forepaw clonus, motor stereotypy, and wet-dog shakes. Overt motor convulsions were characterized by rhythmic clonic jerks of head and forepaws, rearing, salivation and Straub tail. Non-soman control rats showed no evidence of seizures or convulsions. Visual inspection of ECoG recordings, from representative animals of each group, revealed no evidence of altered seizure activity by 2-APB or DMSO, compared with soman-positive controls.

A 60% survival rate was observed in the soman control group (Table 1), and this rate was relatively similar in the other soman-positive groups, except those that also received 1.0 mL/kg DMSO. Of the 10 rats that received 1.0 mL/kg DMSO (with and without 2-APB), only 2 survived; survivors included 1 rat that also received 10 mg/kg 2-APB (i.e., 1 of 3 rats), and 1 rat in the soman-treated 1.0 mL/kg DMSO vehicle control group (i.e., 1 of 4 rats). All 3 rats died that received 22.5 mg/kg 2-APB in 1.0 mL/kg DMSO.

Histopathological evaluations of MAP-2 and H&E-stained brain sections, from all surviving soman-exposed animals irrespective of treatment, revealed severe region-specific brain damage. Damage was bilaterally symmetrical and characterized by widespread tissue necrosis, neuronal loss, chromatolysis, vacuolization, pyknosis and



gliosis. Between bregma -2.64 mm and -3.48mm, severe damage was consistently observed in the piriform and entorhinal cortices, dorsal endopiriform nucleus and the laterodorsal thalamic nucleus. Pronounced damage was often seen in the perirhinal cortex, amygdaloid complex, hippocampus, midline thalamic nuclei, and ventrolateral thalamic nuclei. The pattern of soman-induced seizure-related brain damage (SRBD) seen in the present study is consistent with previous reports (e.g., Petras, 1981; Lemercier *et al.*, 1983; Pazdernik *et al.*, 1985; Carpentier *et al.*, 1990; Ballough *et al.*, 1995, 1998; McDonough *et al.*, 1998). Non-soman, 2-APB/DMSO control rats showed no evidence of neuropathology.

Histopathological damage ratings for H&E-stained brain sections are based on the presence of necrotic neurons and/or absence of a defined neuronal population. Dark shrunken neurons are considered the result of artifactual change. Damage to the neuropil is progressively greater as ratings increase from “mild” to “severe,” and is characterized by increasingly severe malacia and hyalinization typical of necrosis. Group means for H&E damage ratings, as well as cross-sectional areas of necrosis are presented in Table 1. (see below)

Most apparently, the present study demonstrates an exacerbation of soman-induced SRBD by the DMSO vehicle. Every surviving subject, belonging to both DMSO vehicle control groups (i.e., a total of 8 individuals), exhibited "severe" temporal lobe neuropathology (i.e.,  $4.00 \pm 0.00$ ) compared to the below "moderate" average seen in the soman control group (i.e.,  $2.58 \pm 0.27$ ). This difference was very significant ( $p = 0.02$ ) in the 0.7 mL/kg DMSO group. In addition, and despite its unacceptably small group size for statistical comparisons ( $n = 3$  survivors), the 55.4% increase in mean area of temporal lobe necrosis seen in the 0.5 mL/kg DMSO group (i.e.,  $8.39 \pm 1.59$ ) was also significantly elevated above the soman control mean ( $5.40 \pm 0.53$ ;  $p < 0.05$ ).

In the two major groups that received 2-APB (i.e., 13.7 and 5.0 mg/kg), temporal lobe damage was not reduced compared to soman controls (Table 1). In contrast, a very significant elevation in temporal lobe damage (i.e., H&E ratings) was seen in the 13.7 mg/kg 2-APB + 0.7 mL/kg DMSO group (i.e.,  $4.00 \pm 0.00$ ;  $p = 0.01$ ) compared to soman controls (i.e.,  $2.58 \pm 0.27$ ). Interestingly, the 5.0 mg/kg 2-APB + 0.5 mL/kg DMSO group did not show an elevation in either determinant of temporal lobe pathology (i.e., damage ratings or necrosis) compared to soman controls. In fact, the 5.0 mg/kg 2-APB + 0.5 mL/kg DMSO group was the only group in which the combination of soman and DMSO did not result in a "severe" H&E damage rating for absolutely every individual.

No temporal lobe damage was seen in either of the non-soman groups, including the non-soman group that received 22.5 mg/kg 2-APB + 1.0 mL/kg DMSO. This soman-negative control group was created early in the study, before it was suspected that DMSO exacerbated soman-induced SRBD (see above). In light of the fact that no mortality was seen in this group, and there was no evidence of neuropathology, it was deemed an unnecessary allocation of animals to create non-soman control groups for the lower dosages of 2-APB and DMSO.

## DISCUSSION

The present study was unable to provide strong evidence of neuroprotective efficacy by 2-APB in the alleviation of temporal lobe brain damage resulting from soman-induced seizures and *status epilepticus*. However, in one group of soman-treated animals (i.e., those receiving 5.0 mg/kg 2-APB + 0.5 mL/kg DMSO), 2-APB did appear to exhibit a protective effect (see below). In contrast, this study provides unmistakable evidence that the DMSO vehicle (0.5 - 1.0 mL/kg, i.p.) exacerbated neuropathology resulting from soman-induced seizures. For example, every survivor belonging to the soman-positive vehicle control groups (i.e., the 0.7 and 0.5 mL/kg DMSO) exhibited "severe" piriform cortical damage upon H&E neuropathological evaluations (i.e.,  $4.00 \pm 0.00$ ). This represented a very significant increase ( $p = 0.02$ ) in piriform cortical damage compared to the soman control mean that fell below the "moderate" designation (i.e.,  $2.58 \pm 0.27$ ). Consistent with the H&E findings, a 55.4% increase ( $p < 0.05$ ) in the mean cross-sectional area of temporal lobe necrosis (i.e., MAP-2 negative immunostaining) was seen in the soman group that received 0.5 mL/kg DMSO group, compared to the soman control group mean. At the highest dosage (i.e., 1.0 mL/kg), DMSO likely increased mortality in soman-treated groups. While the survival rate remained relatively close to 60%, in the soman control group as well as soman groups that received 0.5 or 0.7 mL/kg DMSO with or without 2-APB, only two rats survived from the soman groups that received 1.0 mL/kg DMSO with or without 2-APB.

The present findings, that DMSO contributed to brain pathology resulting from soman-induced seizures, are difficult to reconcile in light of its widespread use and well characterized effects in mammalian systems. Early toxicological studies indicate that rats can tolerate chronic administration of 9 mL/kg/day DMSO before showing any signs of toxicity (Noel *et al.*, 1975). There have been numerous reports of free radical scavenging and other beneficial effects of DMSO, in rats, at dosages between 1.0 and 6.0 mL/kg (e.g., Wang *et al.*, 2000; Lind *et al.*, 2000; Chang *et al.*, 2001; Nakamuta *et al.*, 2001). Moreover, DMSO toxicity has not been observed in previous studies involving nerve agent administration (e.g., Bodjarian *et al.*, 1995). In 1966, Loomis and Johnson reported a "reversal of soman-induced effects on neuromuscular function with oximes in the presence of dimethyl sulfoxide." On the other hand, it was recently reported that DMSO produces osmotically induced nerve structural changes, ion channel block, and membrane fluidity changes (Larsen *et al.*, 1996). With respect to the osmotic effects of DMSO, it was reported in 1972, by De Bruijne and Van Steveninck, that DMSO can induce osmotic swelling and lysis in various cell types. Alternatively, Kubota *et al.* (1998) reported an enhancing effect of DMSO on neural transmission that was not explained by osmotic effects or blockade of potassium channels, but rather by inhibition of cholinesterase activity or other actions involved in increasing transmitter release from nerve endings. In the present study, it is also possible that DMSO displaced soman that was absorbed into body fat, rendering proportionately more soman available to affect the central nervous system (personal communication from MAJ J.S. Estep, DVM DACVP). Irrespective of whether DMSO increased necrosis by (1) potentiating osmotic swelling and lysis in neurons already rendered vulnerable by metabolic compromise and ionic imbalances, (2)

enhancing cholinesterase inhibition, (3) exacerbating excitotoxicity by facilitating neuronal firing, or (4) effectively increasing soman dosage by displacement from body fat, the present findings give reason for caution when using this vehicle in nerve agent models.

For the soman group that received 13.7 mg/kg 2-APB + 0.7 mL/kg DMSO, piriform cortical damage was very significantly elevated above soman controls (i.e.,  $4.00 \pm 0.00$  vs.  $2.58 \pm 0.27$ , respectively,  $p = 0.01$ ). However, in light of the fact that the soman-positive vehicle control group, which received 0.7 mL/kg DMSO, also had a mean H&E neuropathology rating of  $4.00 \pm 0.00$ , it is entirely possible that the DMSO vehicle is responsible for 100% of the neuropathology seen in the former 13.7 mg/kg 2-APB group.

Interestingly, piriform cortical damage seen in the soman group that received 5.0 mg/kg 2-APB + 0.5 mL/kg DMSO was not elevated above soman controls (i.e.,  $3.00 \pm 0.45$  vs.  $2.58 \pm 0.27$ ). In fact, 3 of the 6 surviving rats belonging to this group exhibited only "mild" piriform cortical damage (i.e., H&E ratings of "2"). This is remarkable considering that all of the other 19 survivors from all soman groups that received DMSO (0.5 - 1.0 mL/kg, with or without 2-APB), including the 0.5 mL/kg DMSO vehicle control group, exhibited "severe" piriform cortical damage. It appears that 5.0 mg/kg 2-APB was able to block the neuropathological effect of 0.5 mL/kg DMSO at the lowest dosage employed in the present study. Further evidence of possible neuroprotective efficacy, by 2-APB, is demonstrated by the significant reduction ( $p < 0.05$ ) in temporal lobe necrosis that is apparent when combined 2-APB (5.0 - 13.7 mg/kg in DMSO) groups are compared to combined DMSO (0.5 - 0.7 mL/kg) vehicle control groups. It is possible that TRP channel inhibition as well as IP3 receptor antagonism, by 2-APB, provided some measure of neuroprotection against the combined soman/DMSO insult.

Despite what appears to be a modest neuroprotective effect by 2-APB, the presence of extensive residual brain damage, in the 2-APB-treated groups, is not encouraging. Although these findings do not support further consideration of 2-APB, as a neuroprotective candidate following nerve agent intoxication, they also do not preclude the possibility that other TRP channel antagonists could prove more efficacious than 2-APB. In fact, very recent evidence indicates that 2-APB selectively blocks the specific subclass of TRP channels known as canonical TRP (TRPC) channels (Sekaran *et al.*, 2007), which have a dense corticolimbic expression pattern in the rat brain and are thought to play a role in learning and memory (Fowler *et al.*, 2007). On the other hand, the melastatin subclass of TRP channels (TRPM) is more strongly implicated in neuropathology resulting from delayed calcium overload (Aarts and Tymianski, 2005). This new evidence may suggest the modest neuroprotection observed in the present study was disproportionately dependent on 2-APB's ability to strongly inhibit IP3 receptors, rather than its effect on TRPC channels. If so, it would be tempting to investigate a possible synergistic interaction, between 2-APB and atropine sulfate, in blocking seizure induction by soman. While atropine sulfate would characteristically block muscarinic receptor overstimulation, 2-APB would block the resultant IP3 elevations immediately down stream.

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## TABLES

**Table 1**  
**Histopathological Damage Ratings and Survival**

Treatment Groups	N (surv/total)	H&E (Mean Rating)	MAP-2 Necrosis (Mean Area; mm <sup>2</sup> )
<u>Soman Groups - Major:</u>			
Soman Controls	18/30	<u>2.58 ± 0.27</u>	<u>5.40 ± 0.53</u>
2APB (13.7mg/kg), DMSO (0.7 mL/kg)	7/11	<u>4.00 ± 0.00*</u>	5.69 ± 0.81
2APB (5.0mg/kg), DMSO (0.5 mL/kg)	6/10	3.00 ± 0.45	5.41 ± 0.37
DMSO (0.7 mL/kg)	5/6	<u>4.00 ± 0.00*</u>	6.66 ± 0.85
DMSO (0.5 mL/kg)	3/4	4.00 ± 0.00	<u>8.39 ± 1.59*</u>
<u>Soman Groups - Pilot:</u>			
2APB (22.5 mg/kg), DMSO (1.0 mL/kg)	0/3	N/A	N/A
2APB (10.0 mg/kg), DMSO (1.0 mL/kg)	1/3	not measured	not measured
DMSO (1.0 mL/kg)	1/4	4.00 ± N/A	8.56 ± N/A
<u>Non-Soman Groups:</u>			
2APB (22.5 mg/kg), DMSO (1.0 mL/kg)	3/3	0.00 ± 0.00	0.00 ± 0.00
Untreated Controls	6/6	0.00 ± 0.00	0.00 ± 0.00

Survival and Histopathology: For H&E histopathology ratings (0-4; where, "0" indicates no damage and "4" maximal), group comparisons were performed using the Mann-Whitney U nonparametric test for comparing central tendency between two groups. For cross-sectional areas of temporal lobe necrosis (MAP-2 negative areas), each experimental group mean was compared against the "soman control" group mean using Dunnett's ANOVA. Underlined means, indicated by asterisks, indicate significant differences (p<0.05) between respective experimental and soman control groups.

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